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Identification of Ser-543 as the Major Regulatory Phosphorylation Site in Spinach Leaf Nitrate Reductase

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Spinach leaf NADH:nitrate reductase (NR) responds to light/dark signals and photosynthetic activity in part as a result of rapid regulation by reversible protein phosphorylation. We have identified the major regulatory phosphorylation site as Ser-543, which is located in the hinge 1 region connecting the cytochrome *b* domain with the molybdenum-pterin cofactor binding domain of NR, using recombinant NR fragments containing or lacking the phosphorylation site sequence. Studies with NR partial reactions indicated that the block in electron flow caused by phosphorylation also could be localized to the hinge 1 region. A synthetic peptide (NR6) based on the phosphorylation site sequence was phosphorylated readily by NR kinase (NRk) in vitro. NR6 kinase activity tracked the ATP-dependent inactivation of NR during several chromatographic steps and completely inhibited inactivation/phosphorylation of native NR in vitro. Two forms of NRk were resolved by using anion exchange chromatography. Studies with synthetic peptide analogs indicated that both forms of NRk had similar specificity determinants, requiring a basic residue at P-3 (i.e., three amino acids N-terminal to the phosphorylated serine) and a hydrophobic residue at P-5. Both forms are strictly calcium dependent but belong to distinct families of protein kinases because they are distinct immunochemically.

INTRODUCTION

Nitrate reductase (NR; EC 1.6.6.1) catalyzes the rate-limiting step in nitrogen assimilation, which is the reduction of nitrate to nitrite by using NAD(P)H as the physiological electron donor (Solomonson and Barber, 1990). The enzyme is cytosolic and has been shown to be a homodimer composed of 110- to 115-kD subunits. Each subunit contains one flavin adenine dinucleotide (FAD), one Fe-heme (cytochrome b_{557}), and one molybdenum-pterin cofactor (MoCo) prosthetic group, which, in that order, transfer electrons from NADH to nitrate (Hoff et al., 1992). Discrete functional domains are associated with the different redox centers and are linked together by hinge regions (Campbell and Kinghorn, 1990). In vitro, NR partial activities can be assayed by using alternate electron donors and acceptors that require only one or two of the enzyme's prosthetic groups.

NR is subject to multivalent control at the level of gene expression (Crawford and Arst, 1993; Crawford, 1995) and post-translational modification (Kaiser and Huber, 1995) in response to environmental factors. Results from several laboratories have indicated that the rapid post-translational modulation involves reversible protein phosphorylation (Kaiser and Spill, 1991; Huber et al., 1992; MacKintosh, 1992). In leaf extracts, phospho-NR (which occurs in the dark) is sensitive to Mg^{2+} inhibition, whereas dephospho-NR (which occurs in the light) is completely unaffected by Mg^{2+}. Thus, assaying the enzyme in the absence of Mg^{2+} represents total potential NR activity, whereas assay in the presence of Mg^{2+} reflects alterations related to the phosphorylation/dephosphorylation mechanism. The latter activity is thought to reflect more accurately in vivo enzyme activity because the cytosol contains free Mg^{2+}.

Spinach leaf NR is ^{32}P-labeled exclusively on seryl residues in vivo when leaves are fed ^{32}P-Pi. Two-dimensional peptide mapping indicated that there are several phosphorylated tryptic fragments and that the phosphorylation status of two of these fragments is correlated with sensitivity to Mg^{2+} inhibition (Huber et al., 1992). NR protein kinase (NRk) has been partially purified and was identified as a calcium-dependent and metabolite-regulated protein kinase (Bachmann et al., 1995). Recent results suggest, however, that phosphorylation of NR is necessary but not sufficient to render the enzyme inactive. An inhibitor protein (IP), which apparently binds to phospho-NR and in the presence of Mg^{2+} inactivates the enzyme, has been partially purified (Spill and Kaiser, 1994; Bachmann et al., 1995; Glaab and Kaiser, 1995; MacKintosh et al., 1995).

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It has been proposed that in this multicomponent process of NR inactivation, IP binds stoichiometrically and thereby inactivates phospho-NR, whereas phospho-NR alone is active catalytically (Glaab and Kaiser, 1995; MacKintosh et al., 1995). Studies using a reconstituted in vitro system have shown that regulation by metabolites and ions of the overall inactivation process seems to be at the phosphorylation level rather than at the interaction of phospho-NR with IP (Bachmann et al., 1995). Calcium activates and phosphate esters inhibit NRk and therefore could play a key role in the post-translational regulation of NR activity in vivo.

To understand better how phosphorylation inhibits enzymatic activity, it is important to identify the regulatory phosphorylation site(s) in the NR molecule. Knowledge of the regulatory phosphorylation site also would permit future attempts to manipulate NR activity by site-directed mutagenesis. Because pure NR is difficult to obtain in large amounts from natural sources and recombinant expression of the active holoenzyme is just now being achieved, recombinant fragments of NR have proved useful for studying the enzyme's biochemistry.

Three recombinant fragments of NR from various organisms and clones have been expressed in *Escherichia coli* (Hyde and Campbell, 1990; Cannons et al., 1991; Campbell, 1992). The most completely studied is the FAD-containing cytochrome *b* reductase fragment (CbR) of maize leaf NR, a 30-kD protein with NADH-dependent ferricyanide reductase activity with derived three-dimensional crystal structure (Lu et al., 1994). A larger fragment of spinach NR, namely, the cytochrome *c* reductase fragment (CcR), consists of the cytochrome *b* and the CbR fragments. The CbR and CcR fragments together with the MoCo binding fragment (Hyde et al., 1991) cover nearly the entire backbone of the enzyme's protein in distinct and defined sections. These fragments were particularly useful to narrow down the portion of NR that contains the regulatory phosphorylation site by using them as "natural" substrates for NRk. Thus, synthetic peptides containing potential target serine residues designed from the spinach NR sequence could be used with confidence to characterize NRk more fully.

In this study, we report the identification of the major regulatory phosphorylation site of spinach leaf NR. We demonstrate its in vitro phosphorylation by a calcium-dependent protein kinase (CDPK), suggesting that NR activity in vivo may be controlled by the concentration of cytosolic free calcium.

RESULTS

Identification of Phosphorylation Sites by Using Recombinant NR Fragments

Earlier studies have shown that NR is phosphorylated exclusively on seryl residues (Huber et al., 1992); the specific residues phosphorylated, however, have not been identified. As one approach to identify the NR phosphorylation sites, different NR fragments were tested as substrates for NRk in vitro. The 30-kD CbR, the 48-kD CcR(+H1), and the MoCo binding fragment were incubated with partially purified NRk in the presence of γ-^{32}P-ATP. The polypeptides were separated by SDS-PAGE, and incorporation of radioactivity was monitored by subsequent autoradiography. Of the three recombinant fragments, which together encompass nearly the entire NR molecule, only the CcR(+H1) fragment was ^{32}P-labeled (data not shown). In addition, only the CcR(+H1) fragment competed with native NR for ATP-dependent inactivation (data not shown). These results suggest that at least one of the phosphorylation sites is contained within the CcR(+H1) fragment, which contains the heme binding domain, the FAD binding domain, as well as the majority of hinge 1 (which connects the heme binding and the MoCo binding domains).

In a subsequent study, incorporation of phosphorous-32 into the CcR(+H1) fragment and a slightly shorter cytochrome *c* reductase fragment CcR(-H1), which is missing a part of the hinge 1 region (residues 531 to 546; ISDSGPTLKRTASTPF), was investigated in more detail (Figure 1). The longer CcR(+H1) fragment was produced by expression in *E. coli*, whereas the small fragment was produced by expression in yeast (Figure 1B). In the eukaryotic system, expression begins at the first Met residue, which corresponds to residue 547 of the spinach sequence, and thus produces a slightly truncated fragment. The CcR(+H1) fragment was heavily labeled in a time-dependent manner, whereas the smaller CcR(−H1) fragment showed no ^{32}P-labeling (Figure 1A). These results suggest that at least one of the NR phosphorylation sites was localized in the N-terminal 16 amino acids of the hinge 1 region that are contained in the CcR(+H1) fragment but are missing from the CcR(−H1) fragment. Because phosphorylation occurs exclusively on seryl residues, potential phosphorylation sites are Ser-532, Ser-534, and Ser-543. Phosphorylation of the CcR(+H1) domain was completely inhibited in the presence of 200 μM EGTA, indicating a Ca^{2+} dependence of the phosphorylation (Figure 1A; see below for further discussion). Phosphorylation of the CcR(+H1) fragment was investigated only qualitatively. However, for a similar experiment using native NR, the stoichiometry of NR phosphorylation was found to approach 1 mol of incorporated phosphorous-32 per mol of inactivated NR subunit (by using data from Bachmann et al., 1995).

Identification of Phosphorylation Sites by Using Synthetic Peptides

An independent approach also was taken to localize further possible phosphorylation sites on NR. Three guidelines were used to identify possible target residues. First, the phosphorylated seryl residue should be conserved among species because regulation of NR activity by protein phosphorylation seems to be widespread and of general importance (Kaiser and Huber, 1995). Second, the surrounding sequences also should contain conserved basic amino acids on the N-terminal side of the phosphorylatable seryl residue as possible recog-

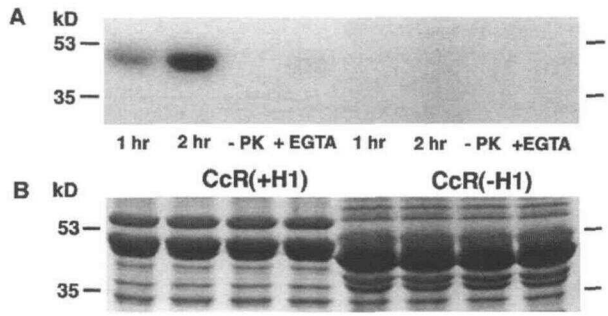

Figure 1. In Vitro Phosphorylation of CcR(+H1) and CcR(−H1).

A recombinant cytochrome *c* reductase fragment, CcR(+H1) (residues 531 to 926 of spinach NR), and a smaller fragment, CcR(−H1) (residues 547 to 926 of spinach NR), were incubated with a 5 to 20% PEG fraction of a crude spinach leaf extract as kinase source in the presence of γ-^{32}P-ATP. As a control, the PEG fraction was replaced with buffer (−PK) or the reaction was performed in the presence of 100 μM EGTA (+EGTA). The reaction was stopped at the time indicated by the addition of trichloroacetic acid.

(A) Detection of the radioactivity incorporated into the NR fragments CcR(+H1) and CcR(−H1) after SDS-PAGE. Radioactivity was localized by autoradiography. Molecular masses are given at left in kilodaltons.

(B) SDS-PAGE analysis of the CcR(+H1) and CcR(−H1) reaction mixtures. Proteins were separated on a 12% SDS gel and visualized by Coomassie Brilliant Blue R 250 staining. Molecular masses are given at left in kilodaltons.

nition elements for NRk (Kemp and Pearson, 1990). And third, the sequence of the NR phosphorylation site should be similar to that of the regulatory phosphorylation site of sucrose–phosphate synthase because ATP-dependent NR inactivation was inhibited greatly by a synthetic peptide homolog of the major regulatory phosphorylation site in spinach sucrose–phosphate synthase (McMichael et al., 1995).

From this analysis, a number of possibilities emerged. Various peptides (designated NR1 through NR4) were synthesized and assayed for phosphorous-32 incorporation from γ-^{32}P-ATP by using a 5 to 20% polyethylene glycol (PEG) fraction of a crude spinach leaf extract as a kinase source (Table 1). From the peptides tested, only NR2 was phosphorylated readily. The NR2 peptide also inhibited the ATP-dependent inactivation of native NR, whereas the other peptides had no effect. The NR2 peptide contained several seryl and threonyl residues as possible phosphorylation sites. In a subsequent study, therefore, the N-terminal half of the NR2 peptide and the C-terminal half were synthesized and investigated separately. Only the N-terminal half of the NR2 peptide (GPTLKRTASTPFJNTTSKJ; designated as NR6) was phosphorylated by the crude kinase preparation and potently inhibited inactivation/phosphorylation of native NR in vitro. Phosphoamino acid analysis of in vitro ^{32}P-labeled NR6 revealed only ^{32}P-phospho-Ser (data not shown), suggesting that Ser-543 and/or Ser-551 are potential phosphorylation sites. Interestingly, Ser-543 is contained in the CcR(+H1) fragment (which is phosphorylated by NRk) but is not contained in the shorter CcR(−H1) fragment (which is not phosphorylated). In contrast, Ser-551 is contained in both the CcR(+H1) and CcR(−H1) fragments. These results strongly suggested that Ser-543, contained in the hinge 1 region connecting the heme and the MoCo binding domains, is indeed the phosphorylation site.

Peptide Mapping

To determine whether the phosphorylated residues on NR6 and the CcR(+H1) fragment are identical, peptide mapping was performed. Two-dimensional mapping of tryptic ^{32}P-labeled phosphopeptides derived from the CcR(+H1) fragment resolved two radioactive spots (Figure 2). In a similar experiment, mapping also was performed with the synthetic peptide NR7 (GPTLKRTASTPFMNTTSK), which is similar to NR6 but comprises the native spinach NR sequence with Met rather than norleucine residues. Mapping of the tryptic fragments derived from ^{32}P-labeled NR7 resolved two spots that comigrated with the two spots of the CcR(+H1) map. Thus, it can be concluded that NR7 comprises the same site(s) that was phosphorylated on the CcR(+H1) fragment. Phosphoamino acid analysis of ^{32}P-labeled NR7 phosphorylated in vitro with γ-^{32}P-ATP and NRk demonstrated that only seryl residues were labeled (data not shown). That Ser-551, also contained in NR7, was not phosphorylated was established by the demonstration that a truncated version of NR7, lacking Ser-551, was phosphorylated by NRk in vitro to the same extent as NR7 (data

Table 1. Phosphorylation of Synthetic Peptides and Their Effect on NR Inactivation

Peptides[a]	Inhibition of NR Inactivation[b] (%)	Phosphorylation[c] (pmol/min/mL)
NR1 R_{239}DVLKRCGVJSSLKGALNV	4.5	148
NR2 R_{525}ERHLEISDSGPTLKRTASTPF	30.7	1559
NR3 S_{557}EVKKHTADSAWIVVHGN	1.2	2
NR4 K_{676}LIEKVSLSHDVRRFRFGLPSEDQ	2.2	37
NR5 R_{525}ERHLEISDSGPTLKR	1.8	4
NR6 G_{535}PTLKRTASTPFJNTTSKJ	73.2	2180

[a] Standard single-letter code; J, norleucine.

[b] NR inactivation was determined as ATP-dependent loss of activity in the presence of IP (assayed with Mg^{2+}) and 50 μM purified peptides.

[c] Peptide phosphorylation was performed under standard assay conditions with a 5 to 20% PEG fraction of crude spinach leaf extract as the kinase source.

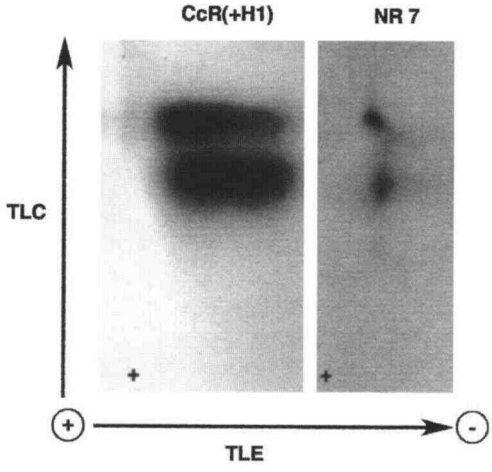

Figure 2. Tryptic Phosphopeptide Maps of in Vitro–Phosphorylated CcR(+H1) and NR7.

The origins are marked with a (+) in the lower left corner of each map. Phosphorylation of NR7 (GPTLKRTASTPFMNTTSK) and the CcR(+H1) domain was under standard conditions. The peptide was passed through an anion exchange column to remove unreacted ATP from the product, whereas the CcR(+H1) domain was electrophoresed and transferred to Immobilon membrane. After digestion with trypsin, the resulting phosphopeptides were separated by two-dimensional thin-layer electrophoresis (TLE)/thin-layer chromatography (TLC). Labeled phosphopeptides were localized by autoradiography.

not shown). Thus, it is most likely that the two spots arose from a single phosphorylation site rather than from multiple sites.

The basis for the resolution of two spots from a single phosphorylation site is not entirely clear. However, artifactual spot "doubling" in the chromatographic dimension, as observed here, has been occasionally observed by others and can be the result of a number of things, including partial oxidation of Met residues (Boyle et al., 1991). To summarize, these results strongly suggest that Ser-543, contained in the hinge 1 region of NR, is the major regulatory phosphorylation site.

NR6 Kinase and NRk Activities Are Identical

To address whether Ser-543 is a regulatory rather than a constitutive phosphorylation site, NR6 kinase (NR6k) activity was partially purified and characterized. Anion exchange chromatography of a 5 to 15% PEG fraction resolved two peaks of NR6k activity eluting at 70 mM NaCl (designated as peak I) and at ~120 mM NaCl (designated as peak II; Figure 3). The two peaks of NR6k activity coeluted with NRk activity assayed as ATP-dependent inactivation of NR. In addition, when peaks I and II were further purified, the elution profiles for NR6k activity and ATP-dependent NR inactivation (i.e., NRk) tracked closely during affinity chromatography on Blue Sepharose, producing in each case a single peak at ~600 mM NaCl (data not shown).

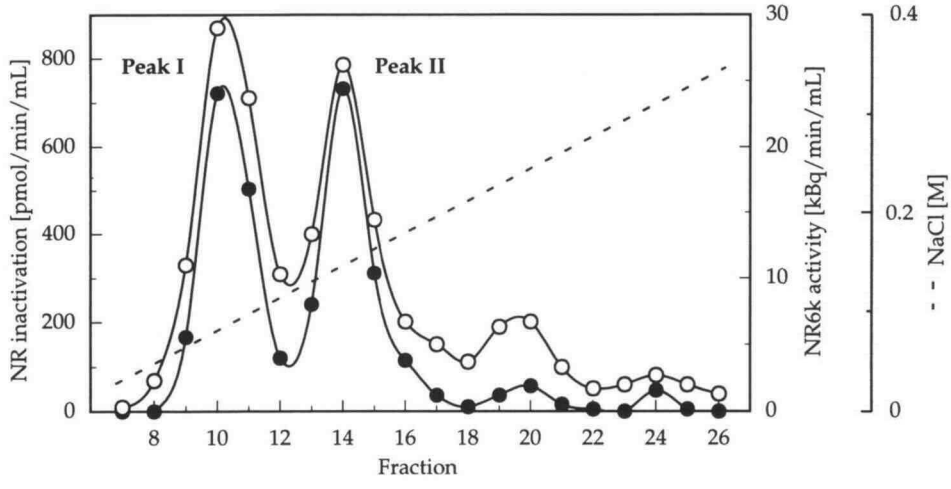

Figure 3. Cochromatography of NRk and NR6k Activities on Resource Q.

Protein of a crude spinach leaf extract precipitating between 5 and 15% PEG was collected by centrifugation and applied on a Resource Q column. After washing with 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, and 2.5 mM DTT, proteins were eluted with a linear gradient from 0 to 500 mM NaCl in buffer, as indicated by the dashed line. NRk activity (●) was assayed as ATP-dependent NR inactivation in the presence of IP and assayed with Mg^{2+}. NR6k activity (○) was assayed as incorporation of phosphorus-32 into the peptide NR6 (GPTLKRTASTPFJNTTSK; J, norleucine) measured by the filter binding assay under standard conditions.

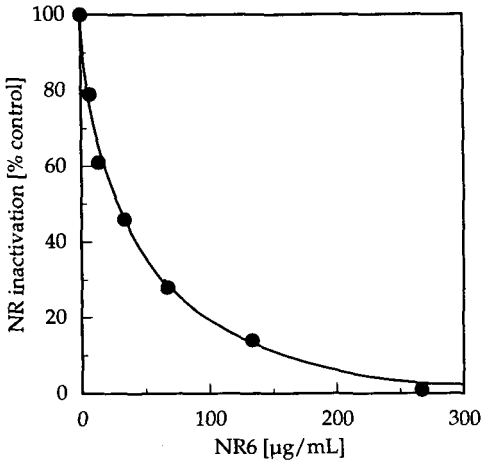

Figure 4. Synthetic Peptide NR6 Prevents the ATP-Dependent Inactivation of Native NR.

NR inactivation was determined as ATP-dependent loss of NR activity in the presence of saturating concentrations of IP and various amounts of NR6 peptide, assayed in the presence of 5 mM $MgCl_2$.

Peaks I and II of NR6k activity showed apparent molecular masses of 45 and 60 kD by using size exclusion chromatography (data not shown). These are the same masses reported previously for the two forms of NRk (McMichael et al., 1995). Further purification of NR6k after anion exchange chromatography was achieved by chromatography on phenyl–Sepharose. Interaction with the hydrophobic matrix of the resin was strong, and NR6k activity could be eluted only with the zwitterionic detergent 3-([3-cholamidopropyl]-dimethylammonio)-1-propane sulfonate (CHAPS). Again, NR6k activity coeluted with NRk activity (ATP-dependent inactivation of NR), suggesting that they are catalyzed by the same protein (data not shown). To substantiate that finding further, competition experiments with NR6 peptide were performed (Figure 4). NR6 inhibited NR inactivation by competing with NR as a substrate for NRk. A 50% inhibition of NR inactivation was found at 25 μg/mL (20 μM), implying a high potency of NR6. To summarize, the persistent copurification of NR6k and NRk activities and the sequence-specific competition of NR6 with native NR for inactivation strongly suggest that Ser-543, located on the synthetic peptide NR6, is the major regulatory phosphorylation site of spinach NR.

NR Partial Reactions

In addition to the full reaction, NR catalyzes a variety of partial reactions in vitro involving one or more of the enzyme's prosthetic groups. Using a variety of electron donors and acceptors allows one to dissect various portions of the overall reaction. Detailed studies were undertaken to identify the site of Mg^{2+} inhibition in the overall nitrate reduction reaction (Table 2). The dehydrogenase activity of phospho-NR with NADH as electron donor and artificial electron acceptors, such as ferricyanide or mammalian cytochrome *c*, was not affected by Mg^{2+}. From all of the partial reactions assayed, only the reduced methyl viologen:NR activity, which requires electron transfer from the heme-iron prosthetic group to the MoCo domain to reduce nitrate, was inhibited by Mg^{2+}. However, the reduced bromphenol blue:NR partial reaction, which requires only the MoCo to reduce nitrate, was unaffected, suggesting that the inhibition of electron flow occurs between the heme and MoCo domains. Furthermore, the Mg^{2+} inhibition was seen only when IP was present, and IP had no effect on the partial reactions in the absence of Mg^{2+}. These results suggest that the interaction of phospho-NR with IP might result in a change of NR conformation that blocks electron flow from the heme to the MoCo domain. These results are certainly consistent with the idea that the regulatory phosphorylation site (Ser-543) is contained in the hinge 1 region.

Characterization of NR6k

To characterize the two kinase forms (peak I and II) further and to gain insight into the nature of the interaction between

Table 2. Partial Reactions of NR Showing the Effect of IP on Dephospho-NR and Phospho-NR Activity in the Presence of Mg^{2+} [a]

Reaction[b]	Dephospho-NR[c] − IP (%)	Dephospho-NR[d] + IP (%)	Phospho-NR − IP (%)	Phospho-NR + IP (%)
NADH:NR	100	98	99	41
NADH:FR	99	98	100	100
NADH:CR	97	98	100	100
MV_r:NR	100	98	99	45
BPB_r:NR	98	100	98	95

[a] Dephospho-NR and IP were purified by PEG fractionation and chromatography on Resource Q. Phospho-NR was prepared by incubating dephospho-NR with 0.66 mM ATP and NRk for 20 min. Partial reactions assayed in the absence of Mg^{2+} showed no difference between dephospho-NR and phospho-NR (data not shown). Enzyme activity (100%) corresponds to 350 nmol/min/mL NO_2^- produced for NADH:NR, 1694 nmol/min/mL NADH consumed for NADH:FR, 2095 nmol/min/mL cytochrome *c* reduced for NADH:CR, 349 nmol/min/mL for reduced methyl viologen:NR, and 2489 nmol/min/mL for reduced bromphenol blue:NR.

[b] FR, ferricyanide reductase; CR, cytochrome *c* reductase; MV_r, reduced methyl viologen; BPB_r, reduced bromphenol blue.

[c] Without IP.

[d] With IP.

Table 3. Kinetic Analysis of Peak I and II Kinase Activities by Using Synthetic Peptide Substrates and Their Effect on NR Inactivation[a]

Peptide Sequence[b]	Peak I Kinase[c] K_m (μM)	Peak I Kinase[c] V_{max} (μU/mL)	Peak I Kinase[c] V_{max}/K_m	Peak II Kinase[c] K_m (μM)	Peak II Kinase[c] V_{max} (μU/mL)	Peak II Kinase[c] V_{max}/K_m	Inhibition of NR Inactivation[d] (%)
NR7 GPTLKRTA**S**TPFMNTTSK	0.8	60.3	75.4	0.6	28.8	48.0	66.9
NR9 GPTLK**A**TA**S**TPFMNTTSK	218.3	40.3	0.2	166.2	9.8	0.1	0.3
NR10 GPTL**A**RTA**S**TPFMNTTSK	2.6	50.7	19.5	2.1	26.4	12.6	36.4
NR11 GPTLKRTA**S**T**A**FMNTTSK	3.6	148.2	41.2	1.6	57.1	35.7	35.5
NR14 GPT**A**KRTA**S**TPFMNTTSK	39.4	14.5	0.4	56.4	2.9	0.1	1.9
NR15 GPTLKRTA**S**TPF**A**NTTSK	8.6	69.3	8.1	6.6	30.1	4.6	34.9
NR13 GPTLKRTA**T**TPFMNTTSK	14.0	157.0	11.2	29.1	21.3	0.7	56.8

[a] Each peptide was purified by HPLC and quantitated by amino acid analysis.

[b] Phosphorylated residues are given in boldface type, and the changes from the peptide NR7 are underlined.

[c] Phosphorylation rates were determined at room temperature under standard assay conditions (see Methods). K_m and V_{max} values were determined by least squares regression fit of a Lineweaver Burk plot.

[d] NR inactivation was determined as ATP-dependent loss of activity in the presence of IP (assayed with Mg^{2+}) and 50 μM purified peptides.

peptide and protein kinase, substrate recognition studies were performed by using synthetic peptides. The possible significance of different residues flanking the phosphorylated seryl residue was analyzed by sequential replacement with alanine residues. Different analogs of peptide NR7 were synthesized and assayed for their ability to serve as substrate for the two enzymes (Table 3).

A single change of the arginine residue at the P-3 position (i.e., three amino acid residues N-terminal to the phosphorylated seryl residue at position 0) to an alanine lowered the V_{max}/K_m ratio 400- to 800-fold, mainly by increasing the K_m value (~270-fold) for both enzymes. The basic residue at P-4 appears to play a minor role in substrate recognition; substitution of alanine for lysine decreased V_{max}/K_m only approximately fourfold. Substitution of alanine for proline at P+2 increased V_{max} slightly, probably due to increased flexibility of the peptide, but also increased K_m slightly. Both peaks I and II recognized hydrophobic residues as specificity determinants at P-5; substitution of alanine for the aliphatic hydrophobic amino acid leucine resulted in a 200-fold reduction of V_{max}/K_m. On the other hand, replacing methionine with alanine at P+4 had little to no effect on the phosphorylation of the peptide. The only significant difference between the peak I and II enzymes was seen when threonine was substituted for serine at the phosphoacceptor site. Whereas a 2.5-fold increased V_{max} was observed for peak I (but with a substantially higher K_m), the peptide was a poor substrate for the peak II enzyme. Thus, both peaks I and II have the minimal recognition motif of hydrophobic-X-basic-X-X-Ser, where X represents any residue.

Inhibition of ATP-dependent NR inactivation by synthetic peptides showed the same sequence specificity as found for the phosphorylation of the peptide analogs (Table 3). No inhibition of NR inactivation was detected at 50 μM peptide concentration when alanine substituted the critical arginine residue at P-3 or leucine at P-5. The peptide NR13 with threonine replacing serine at the phosphorylation site showed a similarly strong inhibition as the original NR7 (56.8% compared with 66.9%).

Are Peaks I and II CDPKs?

The recognition motif basic-X-X-Ser, the ability to bind tightly to a hydrophobic matrix (e.g., phenyl–Sepharose), and the inhibition of activity by micromolar concentrations of EGTA (Figure 1; see also McMichael et al., 1995) implied that peaks I and II could belong to the CDPK kinase family (Roberts and Harmon, 1992). Thus, additional studies were undertaken to investigate the nature of peaks I and II, especially in regard to their relationship with CDPKs. Inhibitor studies included the cyclic nucleotide–dependent protein kinase inhibitors H-7 (which also inhibits protein kinase C activity) and H-8, as well as the CDPK and protein kinase C inhibitor K-252a (data not shown). Both forms of NR6k were potently inhibited by K-252a (75% inhibition at 1 μM), whereas H-7 and H-8 had only little effect (11 to 21% inhibition at 100 μM). As depicted in Figure 5, both forms were strictly Ca^{2+} dependent, with activation at submicromolar concentrations of free Ca^{2+} (half-maximal activity at ~150 nM). Free calcium stimulated the kinase activities 40- to 100-fold. These results clearly support the idea that the peak I and II enzymes belong to the CDPK family.

Immunochemical procedures were used to investigate further the relationship between CDPKs and the two forms of NRk. The spinach leaf enzymes (peaks I and II) and soybean CDPKα (Harper et al., 1991) were immunoassayed by protein gel blotting (Figure 6). The blots were immunostained with a mixture of monoclonal antibodies directed against the catalytic domain of soybean cell CDPKα. Peak II cross-reacted with the

antibodies, producing a single band at ~60 kD, whereas peak I did not. Because the same amount of kinase activity was applied for peaks I and II, there should have been a signal on the blot if peak I was recognized by the antibodies.

In addition, studies using rabbit polyclonal antibodies raised against the calmodulin-like domain (CLD) of soybean cell CDPKα were performed. The peak I and II enzymes were preincubated with increasing amounts of antibodies, and immune complexes were then pelleted after the addition of Immunoprecipitin. The peak I kinase activity, assayed as ATP-dependent inactivation of NR, was not recognized by the antibodies, whereas the peak II enzyme was clearly recognized and immunoprecipitated (Figure 7). Rabbit preimmune serum had no effect on either peak I or II activity (data not shown). The same result was obtained when peak I and II activities remaining in the supernatant were assayed as NR6 phosphorylation (data not shown).

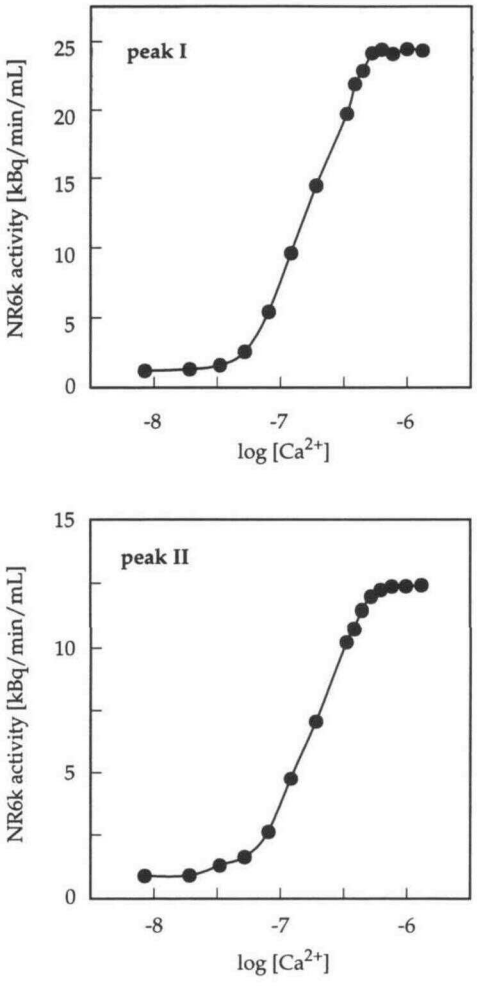

Figure 5. Effect of Free [Ca^{2+}] on Peak I and II NR6k Activities.

Partially purified enzymes were assayed under standard conditions in the presence of 5 mM EGTA and various concentrations of $CaCl_2$ by using the filter binding assay. Free Ca^{2+} concentrations were calculated by using a computer program (Perrin and Sayce, 1967).

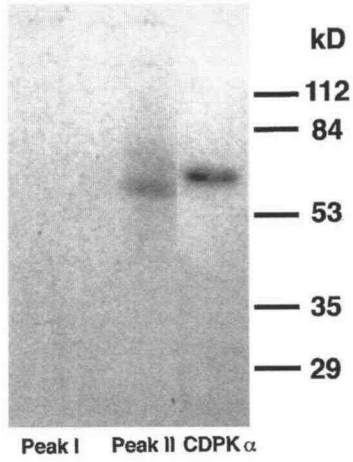

Figure 6. Immunoblot Analysis of Peak I and II Activities.

Partially purified enzymes and CDPKα were electrophoresed on SDS–polyacrylamide gels and electroblotted onto Immobilon-P membrane. The blots were immunostained with a mixture of monoclonal antibodies raised against the catalytic subunit of soybean cell CDPKα. Immunodetection was performed with alkaline phosphatase–conjugated antibodies by using chemiluminescence. Molecular mass markers are given at right in kilodaltons.

DISCUSSION

Identification of the Major Regulatory Phosphorylation Site

Studies using different recombinant NR fragments, which together encompass nearly the entire NR molecule, showed that only the CcR(+H1) fragment was phosphorylated in vitro when incubated with NRk and γ-^{32}P-ATP. However, a slightly shorter fragment CcR(−H1) was not phosphorylated (Figure 1), suggesting that at least one of the NR phosphorylation sites was located within the missing residues, which correspond to a portion of the hinge 1 region located between the heme and the MoCo domains. Synthetic peptides (NR6 and NR7) constituting this portion of the NR molecule were phosphorylated readily by NRk in vitro and inhibited the phosphorylation/inactivation of native NR (Table 1).

Two-dimensional mapping of tryptic ^{32}P-labeled phosphopeptides derived from the CcR(+H1) fragment resolved the same two spots that were obtained from the synthetic peptide (Figure 2), implying that NR7 (essentially the same sequence

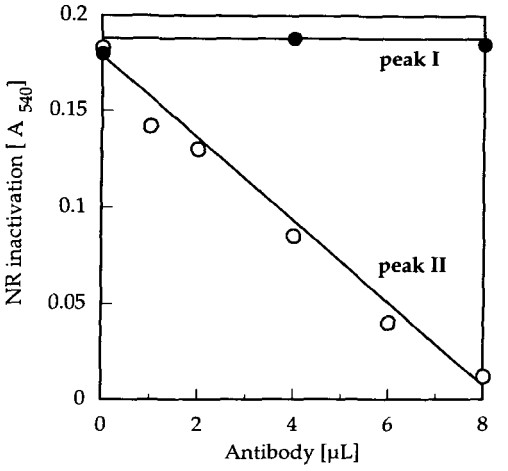

Figure 7. *Immunoprecipitation of Peak II but Not Peak I NRk Activity* by Using Polyclonal Antibodies.

Partially purified peak I (●) and peak II (○) enzymes were incubated with various amounts of polyclonal antibodies raised against the CLD of soybean cell CDPKα. Antibody complexes were immunoprecipitated, and the supernatant was assayed for remaining NRk activity measured as the ATP-dependent inactivation of NR.

as NR6, except methionine replaces norleucine) comprises the same site(s) that was phosphorylated on the CcR(+H1) fragment. Phosphoamino acid analysis showed that NR7 was labeled exclusively on seryl residues, unambiguously demonstrating that either Ser-543 or Ser-551 was the phosphorylated residue. Of these two residues, only Ser-551 is present in the CcR(-H1) fragment, which is not phosphorylated. This result suggests that Ser-543 is the phosphorylation site.

Peptide mapping of spinach NR labeled in vivo separated four phosphopeptide fragments; labeling of two of phosphopeptides was correlated with NR inactivation (Huber et al., 1992). These two phosphopeptide fragments seem to be identical to the ones resolved from NR7 or CcR(+H1) upon comparison of the peptide maps. The appearance of two ^{32}P-labeled phosphopeptide spots on the peptide map is apparently an artifact of spot doubling (Boyle et al., 1991). Thus, phosphorylation of NR7 and CcR(+H1) seems to be correlated to NR inactivation in vivo, implying that Ser-543 is the major regulatory phosphorylation site on NR. Indeed, the elution profiles for the phosphorylation of NR6 and the ATP-dependent NR inactivation tracked closely during anion exchange chromatography, affinity chromatography on Blue Sepharose, and size exclusion chromatography.

From these results, together with the fact that NR6 completely inhibited NR inactivation (Figure 4), it can be concluded that NR6k activity (i.e., phosphorylation of NR6 peptide) and NRk activity (i.e., ATP-dependent inactivation of NR) are catalyzed by the same protein. Consequently, phosphorylation of Ser-543 is directly involved in the process of NR inactivation, and this site is of regulatory significance. Ser-543 also is highly conserved among species; all of the deduced NR sequences from higher plants found in the data base contain a conserved seryl residue at this position as well as flanking residues suggested to be important for recognition by the kinases (Figure 8). Additional studies are necessary to identify the constitutive phosphorylation site that is labeled in vivo (Huber et al., 1992).

Site of NR Inhibition

NR has two active sites: one for electron donation, where FAD is reduced by NADH, and another for reduction of nitrate to nitrite by the MoCo. NR functions as an electron transport system with internal electron transfer from the reduced FAD to the MoCo. Artificial electron donors and acceptors can be used to dissect the overall reaction and to assay partial reactions. The rates of the partial reactions are generally greater than the overall NADH:NR reaction, suggesting that the internal electron transport is rate limiting (Table 2; see also Campbell and Smarelli, 1986). Interestingly, inhibition of NR activity by

Plants	Gene	Residues	Sequence
Tomato	*NIA*	523 - 533	L K K S I **S** T P F M N
Tobacco	*NIA1*	518 - 528	L K K S I **S** T P F M N
Tobacco	*NIA2*	518 - 528	L K K S I **S** T P F M N
Petunia	*NIA*	522 - 532	L K K S I **S** T P F M N
Squash	*NIA*	530 - 540	L K K S V **S** T P F M N
Birch	*NIA1*	515 - 525	L K K S V **S** T P F M N
Arabidopsis	*NIA1*	532 - 542	L K K S V **S** S P F M N
Arabidopsis	*NIA2*	529 - 539	L K K S V **S** T P F M N
Rape	*NIA1*	526 - 536	L K K S V **S** S P F M N
Rape	*NIA2*	526 - 536	L K K S V **S** T P F M N
Soybean	*NIA2*	504 - 514	L K K S V **S** S P F M N
Kidney bean	*NIA1*	502 - 512	L K K S V **S** T P F M N
Kidney bean	*NIA2*	500 - 510	L K K S V **S** S P F M N
Lotus japonicus	*NIA*	508 - 518	L K K S V **S** S P F M N
Cichorium	*NIA1*	521 - 531	L K K S V **S** S P F M N
Maize	*NIA1*	234 - 244	L K R S T **S** T P F M N
Barley	*NIA1*	524 - 534	L K R S T **S** T P F M N
Barley	*NIA2*	521 - 531	L K R S T **S** T P F M N
Rice	*NIA1*	527 - 537	L K R S T **S** T P F M N
Spinach	*NIA*	538 - 548	L K R T A **S** T P F M N

↑

Figure 8. Amino Acid Sequence Comparison around the Putative Regulatory Phosphorylation Site of NR.

The phosphorylated seryl residues are indicated by an arrow, and identical residues at a given position are shown in black boxes with white lettering.

protein phosphorylation appears to occur at the point of internal electron flow from the heme to the MoCo domain, suggesting that the hinge 1 region may be involved. This finding confirmed earlier observations, in which a modulation of the methyl viologen:NR partial reaction by covalent modification was found in crude spinach extracts (Huber et al., 1992). However, no inhibition of NR activity could be seen when assayed in the absence of IP or in the absence of Mg^{2+}. Thus, it can be concluded that the site of NR inactivation (i.e., interaction of IP with phospho-NR in the presence of Mg^{2+}) probably is located between the heme and the MoCo domains.

Interaction of IP with phospho-NR might result in a change or NR conformation, resulting in a block of electron transport. This hypothesis would be consistent with the report that a change in one amino acid in the MoCo domain (from glycine to aspartic acid), which most likely alters enzyme conformation, reduced NR activity and eliminated phosphorylation of the enzyme subunit (LaBrie and Crawford, 1994). Similarly, the deletion of 56 amino acids from the N-terminal domain of the MoCo domain did not affect the kinetic parameters of the enzyme expressed in transgenic tobacco plants (Nussaume et al., 1995). However, post-translational regulation of the enzyme was abolished, most probably because the deletion alters the enzyme's tertiary structure and prevents either the recognition of the Ser-543 for phosphorylation or the interaction of the phospho-NR with IP for inactivation.

Further Purification and Characterization of NRk

Anion exchange chromatography resolved two peaks of NRk activity (Figure 3; Bachmann et al., 1995; McMichael et al., 1995). The two activities were further purified by using phenyl–Sepharose in the presence of Ca^{2+}. The requirement of Ca^{2+} for binding was so low that sufficient Ca^{2+} was inadvertently supplied from the buffer, and no additional Ca^{2+} was needed. Washing the column with high salt (1 M NaCl) or no salt did not elute NRk activity. EGTA (at 1 mM), known to elute CDPKs (Klucis and Polya, 1988; Klimczak and Hind, 1989; Guo and Roux, 1990; Putnam-Evans et al., 1990), did not elute NRk activity either. However, it could be eluted with the zwitterionic detergent CHAPS, demonstrating the strong interaction of the kinase with the matrix. It can be speculated that NRk possesses a hydrophobic domain, similar to calmodulin (Gopalakrishna and Anderson, 1982), which is exposed when calcium is bound. Both forms of NRk are strictly Ca^{2+} dependent, with activation at submicromolar concentrations of free Ca^{2+}, implying that Ca^{2+} might play an important physiological role in the post-translational regulation of NR activity.

Based on the results from the specificity studies (Table 3), we propose that the minimal substrate recognition motif of both peaks I and II is hydrophobic-X-basic-X-X-Ser, where X represents any residue. The primary sequence elements surrounding phosphorylation sites that are important for substrate recognition are usually basic, acidic, or prolyl residues (Kemp and Pearson, 1990; Kennelly and Krebs, 1991). The results obtained with both forms of NRk demonstrated that a hydrophobic residue at P-5 is required for effective substrate recognition. Interestingly, the hydrophobic residue at P+4, which has been shown to be important for recognition by calmodulin-dependent protein kinase I (Lee et al., 1994), was not required with NRk. As shown in Figure 8, not only the phosphorylated seryl residue but also all of the essential recognition elements (i.e., hydrophobic residue at P-5 and basic residue at P-3) were found to be strictly conserved in all of the deduced sequences from several dicots and monocots. This observation suggests that the NRk from other species might be very similar in terms of substrate recognition and probably in other characteristics as well. Indeed, preliminary experiments with other plant species (e.g., squash, wheat, and maize) showed similar NRk elution profiles when subjected to anion exchange chromatography as described for spinach (data not shown). This is consistent with the idea that protein phosphorylation might be a ubiquitous mechanism among higher plants for the regulation of NR activity.

Are Peaks I and II CDPKs?

The minimal recognition motifs found for the peak I and II enzymes are similar to the motifs described for CDPKs (Polya et al., 1989; Roberts, 1989; Putnam-Evans et al., 1990). Protein inhibitor studies and the dependence on submicromolar concentrations of Ca^{2+} for activity suggest that peaks I and II could belong to the CDPK family. Monoclonal antibodies directed against the catalytic domain of soybean cell CDPK cross-reacted with the peak II but not peak I enzyme on immunoblot analysis (Figure 6). However, the antibodies cross-reacted with CDPKs from several plant sources (Roberts and Harmon, 1992), indicating the structural similarities of the enzyme from different species. There are also CDPKs, like the enzymes from Dunaliella (Guo and Roux, 1990), wheat embryo, or Paramecium (Roberts and Harmon, 1992), that do not cross-react with the anti–soybean CDPK antibodies. Whether they all represent a distinct family of CDPK enzymes remains to be determined. The peak II enzyme also cross-reacted with polyclonal antibodies directed against the CLD of soybean cell CDPK, as evidenced by effective immunoprecipitation (Figure 7). Thus, the peak II kinase clearly belongs to the CDPK family and to the same class as the soybean CDPK enzyme. Interestingly, the peak I enzyme did not cross-react with these polyclonal antibodies either. The peak I enzyme has a Ca^{2+} binding domain, as demonstrated by strict dependence on submicromolar $[Ca^{2+}]$; it must be structurally different enough from that of the soybean CDPK enzyme that the antibodies do not recognize the domain. It is intriguing that the two forms of spinach leaf NRk are immunochemically different but show almost identical behavior in terms of Ca^{2+} dependence, substrate recognition, and kinase inhibitor sensitivity.

Because the enzymes differ in size (McMichael et al., 1995), one explanation for the occurrence of two forms of NRk activity might be proteolysis. Peak I, with an apparent molecular mass

of 45 kD, is markedly smaller and could be a proteolytic product of peak II (60 kD). Studies with CDPKs from oat roots and soybean cell cultures showed that the enzyme was subject to proteolysis during extraction (Roberts and Harmon, 1992). Clearly, more work is necessary to determine whether peak I represents part of peak II (and is only an artifact of the extraction) or whether they are distinct enzymes with special functions in vivo. We suspect the latter, because inclusion of a mixture of protease inhibitors during extraction and rapid sample processing has not reduced, or influenced in any way, the recovery of the peak I enzyme (data not shown). We are currently investigating whether the peak I enzyme is a cal- modulin-dependent protein kinase with Ca^{2+} binding elongation factor–hand motifs similar to those of the kinase recently described by Patil et al. (1995).

METHODS

Materials

γ-^{32}P-ATP (111 TBq/mmol) was obtained from Du Pont–New England Nuclear (Boston, MA); microcystin-LR and the protein inhibitors K-252a, H-7, and H-8 came from Calbiochem (La Jolla, CA). Blue Sepharose was made according to the method of Campbell and Smarelli (1978). All other chemicals were from Sigma, unless stated otherwise.

Spinach (*Spinacia oleracea* cv Bloomsdale) was grown in a soil mixture in the greenhouse and fertilized twice a week with modified Hoagland's solution containing 10 mM NO_3^-. Leaves were harvested ~1 hr after the beginning of the photoperiod directly into liquid nitrogen.

Partial Purification of Nitrate Reductase, Inhibitor Protein, and Nitrate Reductase Kinase

Partial purification of nitrate reductase (NR) and inhibitor protein (IP) was performed as detailed by Bachmann et al. (1995). For the kinase purification, deribbed frozen spinach leaves (50 g) were homogenized in 200 mL of extraction buffer containing 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% (v/v) Triton X-100 in a Waring blender. The homogenate was filtered through two layers of cheesecloth and one layer of Miracloth (Calbiochem) and fractionated with polyethylene glycol (PEG). Proteins precipitating between 5 and 15% (w/v) PEG were collected by centrifugation at 38,000*g* for 15 min and resuspended in 10 mL of buffer A (50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, 2.5 mM DTT). After the sample was clarified by centrifugation, it was applied to a 20-mL Resource Q column (Pharmacia). After washing with buffer A, bound proteins were eluted with a 70-mL linear gradient from 0 to 500 mM NaCl in buffer A at a flow rate of 2 mL/min. Active fractions of peak I or peak II were pooled separately and subjected to chromatography on phenyl-Sepharose CL-4B. The 2-mL column was pre-equilibrated with 1 M NaCl in buffer A at a flow rate of 0.5 mL/min and developed at the same flow rate with 15 mL of a linear gradient from 0 to 1 M NaCl in buffer A.

After additional washing of the sample with 15 mL of buffer A, kinase activities were eluted with 10 mL of 0.2% (w/v) 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (CHAPS). Peak fractions were pooled and dialyzed against buffer A for 1 hr. These fractions were used immediately for characterization studies, although the kinase activities were stable for several days when kept on ice. Size exclusion chromatography on a Fractogel TSK HW55 (S) column (Merck, Darmstadt, Germany) was performed as described by Bachmann et al. (1995). For chromatography on Blue Sepharose, kinase fractions were loaded at 1 mL/min onto a 2-mL column pre-equilibrated with buffer A. The column was washed with 12 mL of buffer A at the same flow rate before bound proteins were eluted with a 45-mL gradient of 0 to 1 M NaCl in buffer A.

Enzyme Assays

NADH:NR was assayed as described previously (Huber et al., 1992). To measure reduced methyl viologen:NR and reduced bromphenol blue:NR activities, NADH was replaced by 500 μM methyl viologen or 200 μM bromphenol blue, which were freshly reduced by 5 mM sodium dithionite. The reactions were stopped by air oxidation (vortexing for 30 sec), and nitrate was determined as in the standard procedure. The activity of NADH:ferricyanide reductase was determined spectrophotometrically at A_{340} by using 500 μM potassium ferricyanide and 100 μM NADH in 25 mM phosphate buffer, pH 7.5. To measure NADH: cytochrome *c* reductase activity, the same procedure was used except that ferricyanide was replaced by 1 mg of horse heart cytochrome *c* and the absorption was followed at 550 nm (Hyde and Campbell, 1990).

Nitrate reductase kinase activity was determined as ATP-dependent inactivation of NR activity in the presence of saturating concentrations of IP, assayed in the presence of 5 mM Mg^{2+} (Bachmann et al., 1995). Activity (moles of NR inactivated per minute) was calculated based on the measured enzymatic activity and assuming a specific activity of 100 μmol/min/mg of NR protein (Redinbaugh and Campbell, 1983) under the described assay conditions.

Typical peptide phosphorylation assays consisted of 4 μL of kinase fraction in 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, 0.1 mM γ-^{32}P-ATP (500 cpm/pmol), and 0.1 mg/mL peptide in a total volume of 40 μL. Reactions were initiated by the addition of ATP and incubated at room temperature. After 10 min, 20-μL aliquots were spotted onto Whatman P81 phosphocellulose paper squares (2 × 2 cm) and washed in 75 mM phosphoric acid to remove unincorporated ATP. After four washes of 5 min each, radioactivity bound to the paper was measured by liquid scintillation counting. For the calcium-dependence assay, the mixture contained 5 mM EGTA and various concentrations of $CaCl_2$. Free Ca^{2+} concentrations were calculated by using a BASIC computer program based on a FORTRAN program published by Perrin and Sayce (1967).

Expression of Recombinant NR Fragments

A spinach leaf NR cDNA clone (GenBank accession number U08029) was partially digested with XhoII, yielding 1.2- and 1.6-kb segments encoding the cytochrome *b* reductase fragment (CbR; amino acid residues 673 to 926) and cytochrome *c* reductase fragment (CcR(+H1); amino acid residues 531 to 926). The XhoII fragments were cloned into the BamHI site of *Escherichia coli* expression vector pET3c and expressed by isopropyl β-D-thiogalactopyranoside induction with *E. coli* strain JM109 DE3 (pLysS) (Promega), as described previously for the CbR fragment of maize NR (Hyde and Campbell, 1990). The CcR(+H1) was purified by Blue Sepharose affinity chromatography (Hyde and Campbell, 1990). The 1.6-kb XhoII fragment also was cloned

into the BamHI site of pBluescript KS+ (Stratagene), which permitted the CcR-encoding sequence to be excised with EcoRI. The CcR sequence with boarding EcoRI sites was cloned into the EcoRI site of the *Pichia pastoris* expression vector pHIL-D2 (Invitrogen, San Diego, CA). When the CcR-encoding sequence is expressed in *P. pastoris* by methanol induction, the recombinant protein is made from the first available AUG start codon that results in a CcR fragment without hinge 1 residues (i.e., residues 547 to 926 of spinach NR). When the *P. pastoris*–expressed CcR(−H1) was purified on Blue Sepharose and the purified protein was compared with the *E. coli*–expressed CcR(+H1) by SDS-PAGE, it was clear that the CcR(−H1) was ~3 kD smaller than the CcR(−H1), confirming the predicted absence of the hinge 1 sequence from the *P. pastoris*–expressed CcR fragment.

To prepare the molybdenum-pterin cofactor (MoCo) binding fragment, the squash NR cDNA clone pCmc-3 was restricted with SalI, and the resulting fragment was cloned into the SalI site of the *E. coli* expression vector pET23b (Novagen, Madison, WI). This MoCo binding fragment of squash NR encodes residues 4 to 342 of this enzyme with a predicted molecular size of 42 kD (Hyde et al., 1991). No bromphenol blue:NR activity was found in extracts of *E. coli* expressing the squash MoCo binding fragment, but immunoblotting with antibodies against squash NR confirmed that a protein of the predicted size was present in the extracts.

Phosphorylation of NR Domains

Incorporation of phosphorous-32 into the different NR fragments was performed in a reaction mixture (80 μL) consisting of 12 μg of substrate polypeptide, 10 μL of a 5 to 20% (w/v) PEG fraction of a crude spinach leaf extract as kinase source, 50 mM Mops-NaOH, pH 7.5, 10 mM $MgCl_2$, 0.1 mM γ-^{32}P-ATP (500 cpm/pmol), 0.5 μM microcystin-LR, and 100 μM $CaCl_2$ or EGTA. The reaction was incubated at room temperature and stopped by adding 10 μL of 10% (w/v) trichloroacetic acid at fixed times. After centrifugation at 14,000*g* for 5 min, the resulting protein pellet was washed twice with ice-cold acetone. The pellet was solubilized in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 360 mM 2-mercaptoethanol, 1.5% [w/v] SDS, 7.5% [v/v] glycerol, 0.5% [w/v] bromphenol blue). The polypeptides were separated by SDS-PAGE on a 1.5-mm-thick, 12% acrylamide gel (110 × 140 mm), according to the method of Laemmli (1970). Radioactivity incorporated in the NR fragments was localized by autoradiography with Kodak X-Omat AR film and intensifying screens at −80°C.

Production and Purification of Synthetic Peptides

The peptides NR1 through NR6 were purchased from Chiron Mimotopes Peptide Systems (San Diego, CA). Peptides NR7 through NR15 were synthesized as amides on a peptide synthesizer (model Synergy 432A; Perkin-Elmer) by the solid-phase peptide synthesis process incorporating traditional Fmoc chemistry with 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation. Peptides were cleaved by the trifluoroacetic acid (TFA) procedure after the instructions supplied by the manufacturer. For the kinetic studies, the peptides were purified by chromatography on an Ultremex 5 C18 reverse-phase preparative HPLC column (250 × 10 mm; Phenomenex, Torrance, CA) with a linear gradient from 0 to 40% (v/v) acetonitrile in 0.1% (v/v) TFA at a flow rate of 2.5 mL/min. Purified peptides were dried in a vacuum centrifuge (Heto, Birkerød, Denmark), resuspended in Milli-Q water (Millipore), and dried again to remove residual TFA. Dried peptides were resuspended in Milli-Q water, and an aliquot was quantitated by amino acid analysis with the Waters (Milford, MA) PicoTag chemistry, using the manufacturer's protocol.

Peptide Mapping of CcR(+H1) and NR7

Phosphorylation of NR7 was performed under standard conditions, and the reaction was terminated by the addition of acetic acid (30%). The reaction mixture was passed through an anion exchange column to remove unreacted ATP, and labeled phosphopeptides were eluted with 30% acetic acid (Kemp et al., 1976). The sample was dried in a vacuum centrifuge (Heto) and washed several times with water to remove residual acid. ^{32}P-labeled CcR(+H1) obtained as described above was electrophoresed and transferred to Immobilon-P (Millipore), and the membrane strip containing the labeled CcR(+H1) domain was excised. Digestion of CcR(+H1) and NR7 with trypsin was as described by Huber et al. (1994). The resulting peptides were resolved by two-dimensional thin-layer electrophoresis/thin-layer chromatography (King et al., 1983). Phosphopeptides were identified by autoradiography as described above. Phosphoamino acid analysis with NR7 was performed according to Huber et al. (1994).

Antibody Production

Rabbit polyclonal antibodies directed against the calmodulin-like domain (CLD) of calcium-dependent protein kinase α (CDPKα) from soybean were prepared commercially (Cocalico Biologicals, Inc., Reamstown, PA). The antigen was recombinant CLD (amino acid residues 328 to 508 of CDPKα) expressed in *E. coli* strain BL21 (DE3) pLysS. Cells expressing recombinant CLD were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 20 μg/mL aprotinin) and sonicated. Clarified cell extracts were boiled for 5 min, and denatured proteins were pelleted by centrifugation. The supernatant was brought to 5 mM in $CaCl_2$ and was loaded on phenyl–Sepharose equilibrated with 20 mM Tris-HCl, pH 7.2, and 1 mM $CaCl_2$. After extensive washing with 20 mM Tris-HCl, pH 7.2, and 0.5 mM $CaCl_2$, recombinant CLD was eluted with 20 mM Tris-HCl, pH 7.2, and 2.5 mM EDTA. Pooled fractions from phenyl–Sepharose were applied to Mono Q HR 5/5 (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.2, and 2.5 mM EDTA. Elution was performed with a gradient of 0 to 500 mM NaCl in the same buffer. Fractions containing recombinant CLD were dialyzed extensively against 5 mM NH_4HCO_3 and then against 20 mM Tris-HCl, pH 7.2, and 0.1 mM DTT.

Immunoblots and Immunoprecipitation

Partially purified peaks I and II and CDPKα (Harper et al., 1991) were subjected to SDS-PAGE and electroblotted onto Immobilon-P membrane (Millipore). The protein blots were immunostained with a mixture of monoclonal antibodies (15 μg/mL 14G5 and 5 μg/mL 12G8) raised against the catalytic domain of soybean cell CDPKα (Putnam-Evans et al., 1989). Immunodetection was performed with alkaline phosphatase–conjugated affinity-purified goat anti–mouse IgG and IgN antibodies by using chemiluminescence following the manufacturer's protocol (Tropix, Bedford, MA).

For the immunoprecipitation studies, 20 μL of partially purified peaks I and II was incubated with various amounts of rabbit polyclonal

antibodies raised against the CLD of CDPKα from soybean for 1 hr on ice. Immunocomplexes were precipitated by using 5 μL of 2 × Immunoprecipitin (Gibco BRL) as the precipitating agent. After centrifugation at 14,000*g* for 1 min, the supernatant was assayed for remaining kinase activity under standard conditions.

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